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Atrial natriuretic factor recognizes two receptor subtypes in endothelial cells cultured from bovine pulmonary artery

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In this study specific high affinity binding sites for atrial natriuretic factor (rANF(99–126)) have been identified on cultured endothelial cells of bovine pulmonary artery origin (BPAEC). A time-dependent rise in cellular cGMP levels stimulated by rANF(99–126) was followed by release of the nucleotide into the incubation medium. The use of truncated, ring-deleted and linear atrial peptide analogs in competitive displacement analysis and measurement of cGMP accumulation indicated that only a minor proportion (5–11%) of the available receptor pool was of the ANF-B receptor subtype, linked to guanylate cyclase, with the remaining major proportion possibly of the ANF-C (clearance) receptor subtype. The existence of two ANF receptor subtypes in this cell culture model would suggest a significant role for the circulating peptide in modulation of pulmonary endothelial cell function, which would influence or complement its direct actions on the underlying vasculature of the pulmonary circulation.

ANF receptor subtype; Endothelial cell; Pulmonary artery

1. INTRODUCTION

Atrial natriuretic factor (ANF) is known to influence cardiovascular homeostasis by inducing natriuresis, diuresis and relaxation of vascular smooth muscle [1–3], and as its name suggests, this peptide is primarily synthesised in, stored and secreted from mammalian atria. However, ANF has been detected in low amounts in several other tissues [4–6] and more recently evidence has been provided that the lungs may be capable of ANF synthesis, in particular during the development of cardiac dysfunction [7]. Moreover, ANF receptors have been purified from bovine lung homogenates [8] and experimental studies using isolated arterial and venous preparations have demonstrated that ANF can induce relaxation of the pulmonary vasculature [9,10]. Furthermore, there is evidence that this relaxant effect of ANF is impaired in primary pulmonary hypertension [11], and in pulmonary artery hypertension elevated ANF levels have been reported in the pulmonary circulation [12].

In this study we decided to evaluate the importance of pulmonary vascular endothelium in the actions of ANF since in general, vascular endothelium constitutes the first target 'organ' seen by circulating atrial peptides. The model system which we chose was a primary culture of bovine pulmonary artery endothelial cells (BPAEC). While endothelial cells are known to modulate the responses of isolated blood vessels to a number of vasoactive agents [13], the functional consequences of ANF receptor activation in such cells are a matter for speculation.

We report below on the identification of specific, high affinity binding sites for ANF in cultured BPAEC and we conclude that there are at least two ANF receptor subtypes present in this system, one of which, the ANF-B receptor (present as the minor component) is linked to guanylate cyclase. We further suggest that the so-called ANF-C or clearance receptor which forms the major part of the total receptor pool may be responsible for a time-dependent internalization of ANF observed in these cells. Finally we demonstrate that ANF appears to produce cGMP in BPAEC for subsequent export, since a time-dependent efflux of the nucleotide occurred following challenge with the peptide.

2. MATERIALS AND METHODS

[^{125}I]rANF(99–126) and [^{125}I]NaI (carrier free) both approx. 2000 Ci/mmol were purchased from Amersham. Synthetic peptides rANF(99–126), rANF(101–126), rANF(103–123) and rANF (103–125) were obtained from Sigma. A ring-deleted analog des[Gln^{116},Ser^{117},Gly^{118},Leu^{119},Gly^{120}]ANF102-121-NH$_2$ (C-ANF) and Tyr^{106}rANF(103–125) were obtained from Peninsula Laboratories. The peptides des[Cys^{105},Cys^{121}]rANF104–126 (#SC-46313), and des[Phe106,Gly107,Ala115,Gln116]ANF103–126 (#SC-46542) were a gift from Dr E.H. Blaine. Anti-cGMP serum was obtained from Immunotech, France. All other chemicals were of the highest purity commercially available.

2.1. *Cell culture*

Bovine pulmonary artery endothelial cells (BPAEC), a primary cell line, were obtained from the University of Miami. The cells were

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maintained in DMEM containing 5% foetal calf serum, 5% Nuserum, 50 units/ml of penicillin, 50 μg/ml of streptomycin, 2.5 μg/ml fungizone and 2 mM glutamine at 37°C in a 5% CO_2/humidified incubator. Subcultures were made by passaging confluent cells (which displayed typical cobblestone morphology) with a trypsin (0.5%)/EDTA (0.2%) mixture in Hank's balanced salt solution (HBSS). Typically, passages 11–24 of these cells were used in experiments.

2.2. *Iodination of cGMP*

Iodination of succinyl cyclic GMP tyrosine methyl ester was performed as described by Cailla et al. [14] and the monoiodinated derivative was purified on Sephadex G25. The ability of the antibody to bind the derivative in eluted fractions was tested and those with the greatest affinity for antibody were pooled and used in assays.

2.3. *Binding studies*

All binding assays were performed in 24-well plates (Nunclon) (seeding density approximately 100000 cells/well). Prior to incubations with [^{125}I]rANF(99–126), cell monolayers were washed twice with 1 ml assay buffer (HBSS containing 2 mg/ml bovine serum albumin) at 37°C. Various concentrations (0.05–3.0 nM) of [^{125}I]rANF(99–126) in assay buffer (spec. act. ~450 cpm/fmol) were added to the wells and the plates were placed in a CO_2/humidified incubator at 37°C for 30 min. Cells in parallel wells were exposed to identical concentrations of [^{125}I]rANF(99–126) but with 100-fold molar excess of unlabelled rANF(99–126) added, to determine nonspecific binding. Following the binding reaction, the cells were immediately washed (4 × 1 ml) with ice-cold assay buffer. Cell monolayers were then treated with 700 μl 1 M NaOH at 37°C for 10 min and bound radioactivity was determined using an LKB Universal Gamma counter. Alternatively, 200 μl/well of trypsin-EDTA mixture in HBSS was added to dissociate cells for counting on a haemocytometer. For competitive displacement experiments 0.5 nM [^{125}I]rANF(99–126) was added to the wells alone or in the presence of increasing concentrations (1 nM–1 μM) of unlabelled rANF(99–126), rANF(103–125), #SC-46313, #SC-46542, C-ANF or rANF(103–123). The cells were then incubated and treated as above.

For receptor internalization studies 0.5 nM [^{125}I]rANF(99–126) was added to the wells and the cells were incubated at either 37°C or 4°C for the times indicated. The cells were washed (4 × 1 ml) with ice-cold assay buffer and treated either directly with 700 μl 1 M NaOH as above to determine cell-bound radioactivity, or after 'acid-stripping' with 0.2 M acetic acid pH 2.5, containing 0.5 M NaCl for 6 min at 4°C to remove surface-bound radioactivity (method of Haigler et al. [15]).

2.4. *Intracellular cGMP measurement*

Endothelial cells subcultured in 24-well plates were used. Cell monolayers were first washed (as described above), then preincubated with assay buffer containing 0.5 mM isobutylmethylxanthine (IBMX) for 15 min at 37°C prior to the addition of ANF or its analogs. Following incubation of cells with peptide for the specified times, reactions were stopped by aspiration and samples were then processed in either of two ways: (i) 600 μl 10% (w/v) trichloroacetic acid (TCA) was added to each well and samples were extracted with water-saturated ether (4 × 4 ml) and evaporated to dryness; (ii) wells were treated with 300 μl 0.1 M HCl for 1 h. The amounts of cGMP were quantitated by radioimmunoassay after acetylation, using standard procedures [16].

2.5. *Extracellular cGMP measurement*

At an appropriate time at 250 μl aliquot of incubation medium was taken and treated as described by Schini et al. [17]. Briefly, the sample was extracted with 1.2 M $HClO_4$ and neutralized with KOH before acetylation and radioimmunoassay.

2.6. *Data analysis*

ANF binding data were analysed using the LIGAND computer programme [18] and significance levels were determined using a paired Student's *t*-test.

3. RESULTS AND DISCUSSION

The binding of [^{125}I]rANF(99–126) to BPAEC was saturable, as can be seen in Fig. 1 and Scatchard analysis of the binding data using a one-site model (Fig. 1, inset) indicated the presence of a single class of high affinity binding site for rANF(99–126): K_d and B_{max} values were respectively 0.62 ± 0.08 nM and 102 ± 20 fmol/10^6 cells, corresponding to 52400 sites/cell. Similar K_d values have been reported for other tissues such as rabbit lung membranes [19], bovine aortic endothelial cells and vascular smooth muscle cells [20]. While fitting of a two site model to the binding data generated with rANF(99–126) was not significantly better than the single site model, the existence of receptor subtypes is however suggested by the experiments described below.

Binding of [^{125}I]rANF(99–126) was displaced by unlabelled rANF(99–126), rANF(103–125), #SC-46313, #SC-46542, C-ANF and rANF(103–123) in a dose-dependent manner (Fig. 2), and the comparable IC_{50} values (the concentration of peptide required to displace 50% of the radioligand binding) are shown in Table I. However, in contrast to rANF(99–126) and rANF(103–125), #SC-46313, #SC-46542, C-ANF and rANF(103–123) appeared to incompletely displace [^{125}I]rANF(99–126) binding, even at a concentration of 1 μM. The results indicate that #SC-46313, #SC-46542, C-ANF and rANF(103–123) failed to displace 5%, 11%, 7.8% and 6.5% of binding respectively. A more detailed examination (using an increased number of replicate measurements) of the extent of residual rANF(99–126) binding in the presence of saturating concentrations of #SC-46313, #SC-46542, C-ANF and rANF(103–123) was thus undertaken. The results shown in Table I indicate that each of these ligands displaced significantly less [^{125}I]rANF(99–126) than rANF(99–126) at the same concentration. Furthermore, analysis of the inhibition curves (Fig. 2) using the LIGAND program showed that the data best fitted a one site model i.e., the incomplete displacement is not explained in terms of varying affinity states of the individual ligands for receptor subpopulations. In summary therefore, although all peptides studied bind with comparable affinity, rANF(103–125), #SC-46313, #SC-46542, C-ANF and rANF(103–123) apparently fail to recognise a small proportion of the ANF receptor pool in BPAEC. To further quantify binding of rANF(99–126) to these remaining sites, the displacement of [^{125}I]rANF(99–126) by unlabelled rANF(99–126) was carried out in the presence of an excess of #SC-46542 and #SC-46313. Based on the initial displacement experiments, a concentration of 1 μM #SC-46542 or

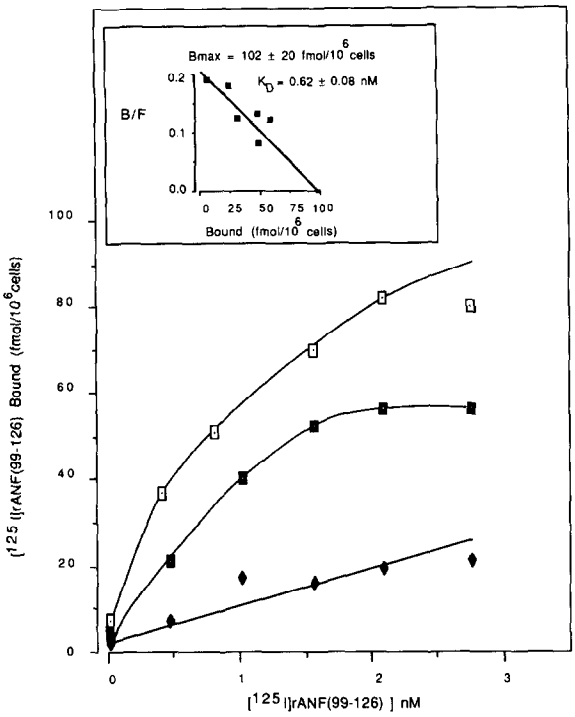

Fig. 1. Saturable binding of [^{125}I]rANF(99–126) to cultured BPAEC. Confluent cells were incubated at 37°C for 30 min with the indicated concentrations of [^{125}I]rANF(99–126). Nonspecific binding (solid diamond), determined in the presence of a 100-fold molar excess of unlabelled rANF(99–126), was 5-20% of the total binding (open boxes). Specific binding (solid boxes). Data points represent mean values from a representative experiment performed in duplicate. (Inset) Scatchard plot of binding data. K_d and B_{max} values given are means ± SE from 4 experiments performed in duplicate.

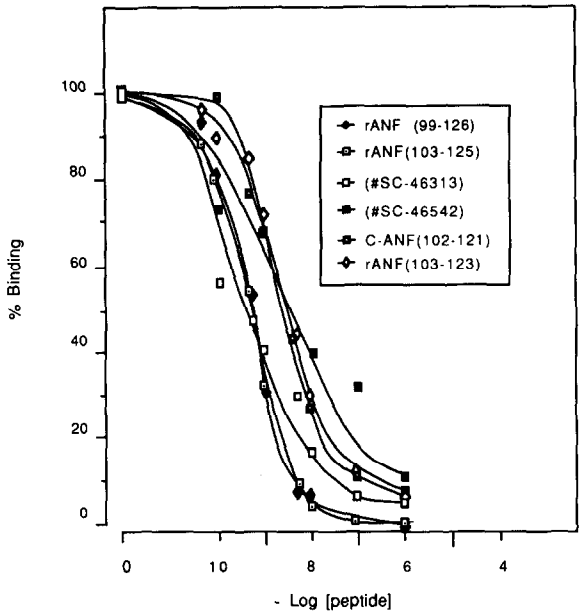

Fig. 2. Competitive displacement of [^{125}I]rANF(99–126) binding by varying concentrations of unlabelled atrial peptides; see inset for key. [^{125}I]rANF(99–126) (0.5 nM) was incubated with confluent monolayers of BPAEC in 24-well multiwell plates and various concentrations of unlabelled analogs. Specific binding data shown were calculated as total binding minus non-specific binding (in the presence of 1 μM unlabelled rANF(99–126)). Data points represent the mean of at least 2 individual experiments, performed in duplicate. For the sake of clarity, standard errors were omitted from the figure but were < 10% of control except for those at 1×10^{-9}, 5×10^{-9} and 1×10^{-8} M rANF(103–123), which were 12.9%, 14.7% and 12.9%, respectively.

#SC-46313 was chosen which would displace the radioligand from at least 90% of the binding sites. Under these conditions, rANF(99–126) completely displaced residual [^{125}I]rANF(99–126) binding (Fig. 3). The IC_{50} for rANF(99–126) was similar in the absence or presence of 1 μM unlabelled #SC-46542 or #SC-46313 (0.4 nM, compared to 0.63 nM and 0.2 nM). This would suggest that rANF(99–126) binds to both classes of binding sites with the same affinity and is nonselective, whereas #SC-46542 and #SC-46313 bind to the major subset (90%/92%) of ANF binding sites. The ability of atrial peptides to induce cGMP accumulation was next investigated and Fig. 4 shows the time-course profile obtained for intra- and extracellular cGMP accumulation in BPAEC stimulated with 0.1 μM rANF(99–126). A similar profile was obtained for rANF(103–125) (data not shown). Intracellular cGMP levels were maximised 1–5 min after exposure of the cells to peptide, thereafter decreasing but still remaining elevated above basal after 2 h. While the effects of raised intracellular cGMP levels on endothelial cell function are not as yet established, it has been proposed that this nucleotide

Table I

	Binding IC_{50} (nM)	Intracellular cGMP accumulation EC_{50} (nM)	% Total binding 1 μM peptide
rANF99–126	0.4	0.10	8.9 ± 1.5
rANF101–126	n.d.	0.10	–
rANF103–125	0.84	3.00	–
Tyr^{106}ANF103–125	n.d.	5.00	–
rANF103–123	4.3	>1000	17.3 ± 2.5**
C-ANF102–121	4.95	>1000	14.6 ± 3.2*
(#SC-46313)	0.42	>1000	16.8 ± 1.2**
(#SC-46542)	3.28	>1000	21.5 ± 4.8**

IC_{50} values (i.e. the concentration of peptide required to displace 50% of the radioligand binding) and EC_{50} values (i.e. the concentration of peptide required to produce half-maximal cGMP accumulation relative to the maximal standard response obtainable with 1 μM rANF(99–126)) for the atrial peptide analogs used. $n \geq 3$ in each case. (n.d. = not determined); % total binding in the presence of 1 μM displacer. Comparisons of residual [^{125}I]rANF(99–126) binding (cpm) were made between rANF(99–126) and (i) #SC-46313, (ii) #SC-46542, (iii) C-ANF and (iv) rANF(103–123) and differences were significant at the * $P < 0.025$ and ** $P < 0.005$ level in each case; data from at least four separate experiments each performed in triplicate

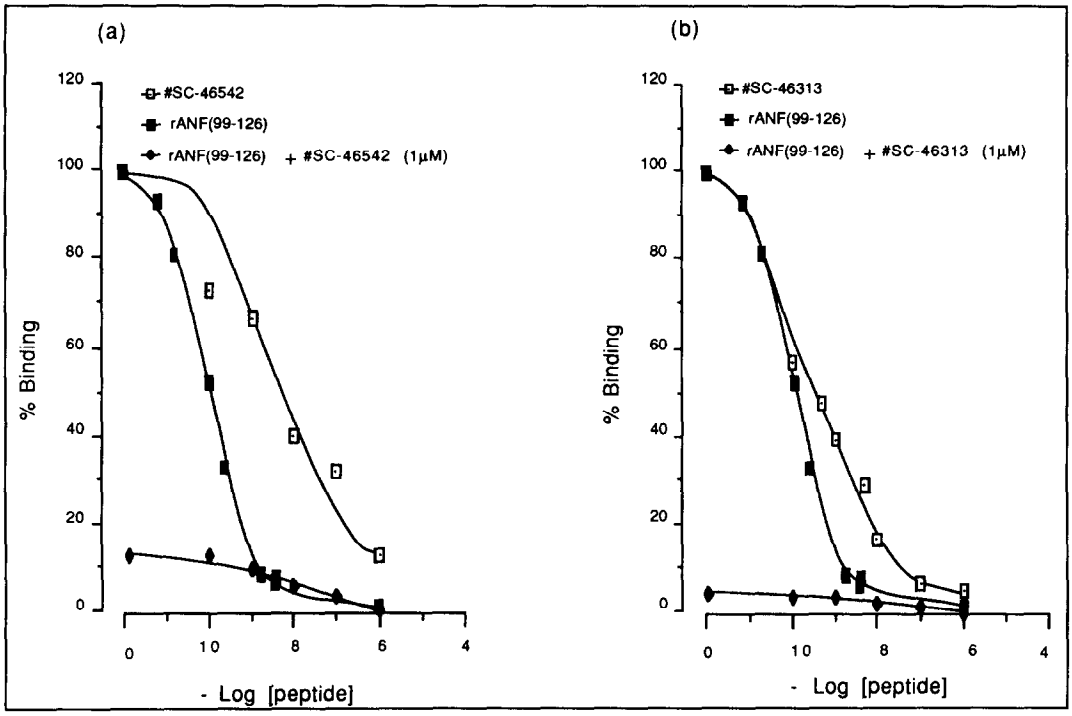

Fig. 3. Competition for [^{125}I]rANF(99–126) binding to BPAEC by rANF(99–126), #SC-46542 (a), #SC-46313 (b), and rANF(99–126) in the presence of 1 μM #SC-46542 (a) or #SC-46313 (b). See insets for key. Binding data were corrected for non-specific binding as detailed in the legend to Fig. 2. The data presented are means of duplicate determinations from one experiment which was repeated three times with similar results. Standard deviations are not shown but were <5% of control except for those at 1×10^{-8} M #SC-46542 and 1×10^{-9} M #SC-46313 which were 7.7% and 9.05%, respectively.

can exert a cytoprotective effect by antagonising the actions of agents known to increase endothelial cell permeability [21]. rANF(99–126)-stimulated production of cellular cGMP was followed by a time-dependent release of the nucleotide into the incubation medium. This release was not significant at short incubation times (when cellular cGMP content peaked), but thereafter increased steadily for up to 2 h.

The association of ANF-stimulated cellular cGMP accumulation with a subsequent time-dependent release of nucleotide into the extracellular medium has been reported in a number of cell types including mammalian erythrocytes [22], glial cells [23], fibroblasts [24], bovine aortic endothelial cells and smooth muscle cells [25]. The role of this released cGMP is not known at present although it is possible that it is merely expelled as part of a mechanism to control cellular nucleotide content. Alternatively however, this released cGMP may somehow itself gain access to the smooth muscle and thereby contribute to vascular relaxation.

In order to compare the ability of ANF and its analogs to stimulate intracellular cGMP accumulation, a series of dose-response relationships was established. The order of potency for the peptides was rANF(99–126) > rANF(101–126) > rANF(103–125) > Tyr^{106}rANF(103–125) >>>> C-ANF, with #SC-46313, #SC-46542 and rANF(103–123) giving no perceptible increase in intracellular cGMP levels above basal (Fig. 5). The relevant EC_{50} values are given in Table I. In addition #SC-46542 and #SC-46313 do not appear to be competitive antagonists at the ANF-B

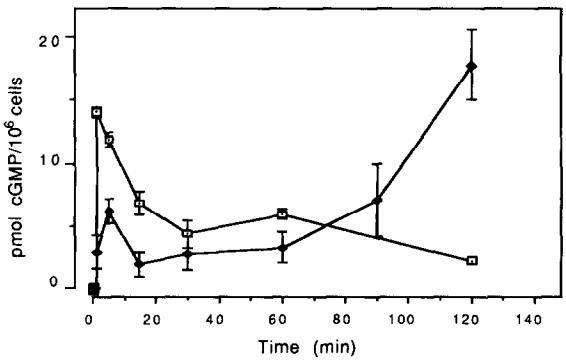

Fig. 4. Time-course profile for intracellular (open symbols) and extracellular (solid symbols) cGMP accumulation in BPAEC stimulated with rANF(99–126). Each data point represents the mean ± SE of three determinations (stimulated – basal). Cells were washed as described in section 2 and incubated for 15 min with 0.5 mM IBMX in assay buffer before the addition of 0.1 μM rANF(99–126) (final conc.). At the specified times an aliquot of incubation medium was taken (for measurement of extracellular cGMP) and the reaction was stopped by aspiration. The cells were treated with either TCA or HCl for extraction of cellular cGMP. In both cases the cGMP was quantitated by radioimmunoassay.

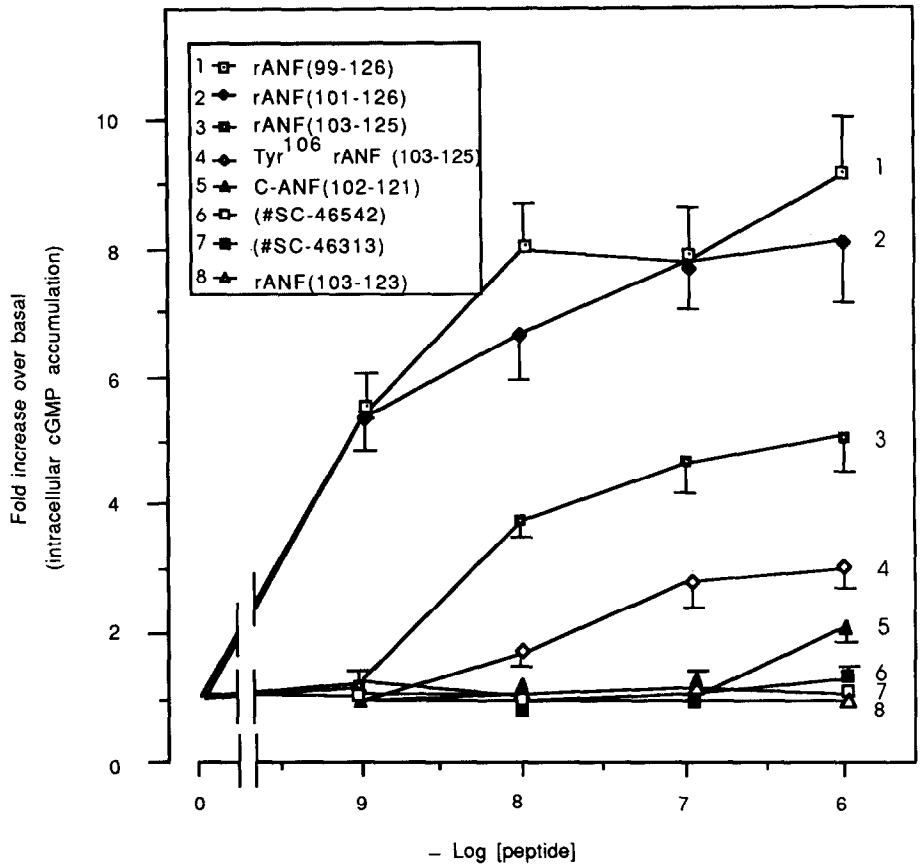

Fig. 5. Dose–response curves for intracellular cGMP accumulation stimulated by the indicated atrial peptides (see inset). Data points represent mean ± SE of three determinations. Cells were preincubated with 0.5 mM IBMX and then with the specified peptide concentrations for 5 min at 37°C. Reactions were stopped by aspiration and cGMP extracted as described in section 2. Basal cGMP accumulation: 1.55 ± 0.15 pmol cGMP/10^6 cells/min (n = 10).

receptor since neither of the two analogs affected the log dose–response curve for cGMP accumulation by rANF(99–126) (data not shown). Basal accumulation of cGMP in these cells was 1.55 ± 0.15 pmol/10^6 cells/min. The magnitude of the ANF-stimulated increases varied considerably between different cell batches but was reproducible within a given batch. In contrast to previous reports of maximal increases in bovine [17] and porcine [26] aortic endothelial cells (160- and 142-fold respectively), the stimulations obtained here are relatively small, maximal increases over basal being 9–12-fold. However, basal cGMP accumulation in BPAEC appears to be relatively high which may reflect a greater metabolic activity of this tissue.

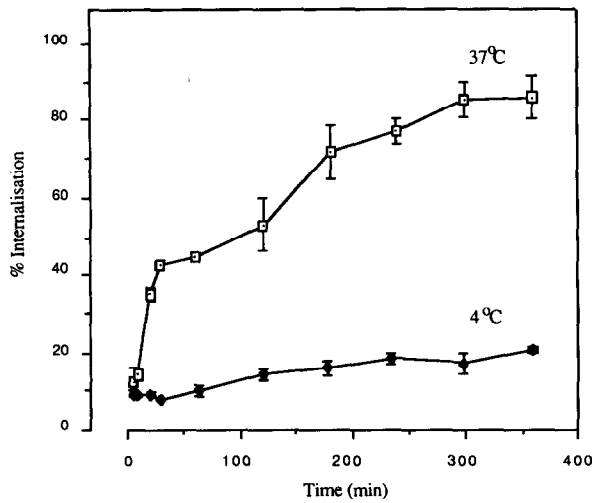

Fig. 6. Time-course for [^{125}I]rANF(99–126) internalization in BPAEC at 37°C and 4°C. Results are expressed as the % of cell-bound radioactivity at each time point which remained following 'acid-stripping'. Each point represents the mean ± SE of two separate experiments performed in duplicate.

To summarise the cGMP data therefore, it appears that those ligands which are selective for the major subset of binding sites are also devoid of guanylate cyclase stimulant activity. This selectivity has previously been reported for #SC-46313 in rabbit lung membranes [27]. Furthermore, the ring-deleted analog C-ANF which produced only a slight increase in cGMP accumulation (~2-fold at 1 μM, see Fig. 5) has been shown to be selective for the ANF-C, or clearance receptor in the kidney and to mediate the sequestration and metabolic clearance of ANF in this organ [28]. It is possible therefore that the major subset of ANF binding sites in BPAEC are ANF-C receptors and that the pulmonary circulation serves an important clearance

function for ANF. Indeed it has been shown in a study involving human subjects that the lung clears more ANF than other tissues such as liver or kidney [29]. In support of such a function, we observed a time- and temperature-dependent internalization of [^{125}I]rANF (99–126) in these cells (Fig. 6), with up to 84% of the cell bound radioactivity located in an acid-resistant (intracellular) compartment after 6 h at 37°C. The overall significance of the lung in ANF action is also supported by the observation that this organ is an important source of the peptide (stored as the 126 amino-acid prohormone) in experimental cardiomyopathy [7].

In conclusion therefore, this study demonstrates for the first time that vascular endothelial cells from the pulmonary circulation express two ANF receptor subtypes. We suggest that interaction of the peptide with both of these receptors may be of primary importance in the actions of ANF in the lung.

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